Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1310–1313, April 1976
Medical Sciences

Human papilloma virus DNA: Physical mapping and genetic heterogeneity

(warts/molecular weight/restriction endonuclease cleavage)

LUTZ GISSMANN AND HARALD ZUR HAUSEN

Institut für Klinische Virologie der Universität Erlangen-Nürnberg, Loschgestr. 7, 852 Erlangen, Federal Republic of Germany

Communicated by Werner Henle, January 14, 1976

ABSTRACT **The molecular weight of three preparations of human papilloma virus DNA derived from different plantar warts was determined by agarose gel electrophoresis or electron microscopic contour length measurement. It was found to amount to approximately 4.9×10^6. Analysis of this DNA after sequential digestion by four different restriction endonucleases (*Eco*RI, *Bam*, *Hin*d II, and *Hin*d III) permitted physical mapping of the cleavage sites. Two of the three DNA preparations revealed an identical cleavage pattern, whereas the third one contained two additional cleavage sites.**

The inoculation of cell-free extracts from common warts (verrucae vulgares), genital warts (condylomata acuminata), and laryngeal papillomas into the skin of human volunteers resulted in the induction of common warts several months after injection (1–3). This led to the assumption that the different clinical pictures were induced by the same agent (4). Some recent observations suggest, however, the existence of strain and type differences among human papilloma viruses: (*i*) the age distribution of patients with verrucae vulgares, genital warts, or laryngeal papillomas differs strikingly (5); (*ii*) immune electron microscopy suggested one-way cross-reactivity between common wart and condyloma viruses (6); (*iii*) complementary RNA prepared with papilloma viral DNA derived from a plantar wart did not hybridize with DNA from condylomata acuminata and laryngeal papillomas (7).

This paper analyzes the restriction endonuclease cleavage pattern of three papilloma viruses isolated from plantar warts. The data indicate genetic heterogeneity even within papilloma viruses purified from histologically similar epithelial proliferations.

MATERIALS AND METHODS

Virus Purification. Plantar warts, stored at −20°, were minced, ground with sterile sea sand in a mortar, and then suspended in 5–10 ml of 1 M sodium phosphate buffer (pH 8.0). The homogenate was treated with trypsin (Difco) at a final concentration of 0.01% for 1 hr at 37°. After centrifugation at $2000 \times g$ for 15 min at 4°, the supernatant was examined by electron microscopy for the presence of papilloma particles. Particle-containing suspensions were pelleted in the Spinco SW 27 rotor (80,000 × *g*, 75 min, 4°). Thereafter, the pellet was resuspended in 3 ml of phosphate buffer containing 0.05 M NaCl, 0.01 M EDTA, and 0.05 M sodium phosphate (pH 7.4). Solid cesium chloride was added to yield a final density of 1.34 g/ml. After equilibrium centrifugation in the Spinco SW 56 rotor (110,000 × *g* for 40 hr at 20°), two clearly visible bands (containing full and empty particles, respectively) were collected separately after piercing the bottom of the tube. The particle-containing fractions were dialyzed against 0.01 M Tris · HCl (pH 8.1) containing 0.001 M EDTA.

Purification of Viral DNA. Papilloma viral DNA was extracted according to Tai *et al.* (8). Sodium dodecyl sulfate (25%) was added to the virus suspension to a final concentration of 1%. The solution was incubated at 37° for 10 min. Thereafter, solid CsCl was added to yield a concentration of 1 M. The solution was then kept for at least 15 min at 0°. The precipitating sodium dodecyl sulfate was removed by low-speed centrifugation at 0°. Ethidium bromide (200 μg/ml) was added and the density was adjusted to 1.56 g/ml. After equilibrium centrifugation in the SW 56 rotor at 110,000 × *g* for 40 hr at 20°, two distinct bands were visualized by UV light at densities of 1.60 and 1.56 g/ml. They were collected separately. Ethidium bromide was removed by three extractions with water-saturated *n*-butanol and two subsequent extractions with ether. The DNA was then dialyzed against 0.01 M Tris · HCl (pH 8.1) containing 0.001 M EDTA.

Viral DNA studied in these experiments originated from three different plantar warts with the laboratory codes WV-432, WV-474, and WV-494.

Restriction Endonuclease Cleavage of Wart Viral DNA. *Eco*RI, *Hin*d II, and *Hin*d III were kindly provided by P. Philippsen, the *Bam* H1 and *Sal* enzymes were a generous gift of C. Mulder.

(*i*) *Eco*RI. Reaction mixtures (0.5 ml) containing 0.1 M Tris · HCl (pH 7.5), 0.01 M $MgCl_2$, 300 ng of viral DNA (Co II), and 0.3 μl of *Eco*RI were incubated for 3 hr at 37°.

(*ii*) *Hin*d II and *Hin*d III. Mixtures (0.5 ml) containing 0.01 M Tris · HCl (pH 7.5), 0.006 M $MgCl_2$, 0.006 M mercaptoethanol, 0.015 M KCl, 300 ng of DNA, and 25 μl of *Hin*d II or 3 μl of *Hin*d III were incubated at 30° overnight.

(*iii*) *Bam* and *Sal*. Samples with 300 ng of DNA and 2 μl of *Bam* or *Sal* were incubated at 37° for 3 hr in the same buffer as described for *Hin*d II and *Hin*d III.

After digestion, the reaction was stopped by adding EDTA to a final concentration of 0.02 M. The DNA was precipitated in a 2.5-fold volume of ethanol and 0.21 M sodium acetate at 0°. The precipitate was collected by centrifugation in the Spinco SW 56 rotor for 30 min at 110,000 × *g* and then suspended in 10–20 μl of 0.1 times the concentration of electrophoresis buffer. This solution was mixed with 10 μl of 40% sucrose, 0.125% bromphenol blue and then applied to the gel.

Gel Electrophoresis. Gel electrophoresis was carried out through 0.7% or 1.0% agarose (Seakem) slab gels in a Hoefer electrophoresis chamber (18 × 14 × 0.3 cm) at 18 V for 18 hr at 4°. Polyacrylamide gel electrophoresis through 3.8% acrylamide, 0.2% *N,N'*-methylenebisacrylamide slab gels (18 × 14 × 0.1 cm) was performed at 100 V for 5 hr at 4°. The electrophoresis buffer contained 0.04 M Tris · HCl (pH

Table 1. Number of fragments after digestion of wart viral DNA with restriction enzymes *Eco*RI, *Bam*, *Sal*, *Hind* II, and *Hind* III

Enzyme	Papilloma DNA isolate		
	WV-432	WV-474	WV-494
*Eco*RI	2	2	2
Bam	1	2	1
Sal	—	—	—
Hind II	3	3	3
Hind III	3	4	3

7.8), 0.005 M sodium acetate, and 0.001 M EDTA. The DNA bands were visualized after staining for 60 min with ethidium bromide (2 μg/ml) under UV light. The photographs were taken with a Polaroid Land camera.

Calculation of Molecular Weight by Gel Electrophoresis. The molecular weights of fragments obtained after restriction endonuclease cleavage were calculated by using PM2 DNA cleaved by *Hind* III (kindly provided by R. E. Streeck) as a length marker. The molecular weights of these fragments are 3.5×10^6, 1.3×10^6, 0.6×10^6, 0.29×10^6, 0.28×10^6, 0.17×10^6, and 0.065×10^6 (Streeck, personal communication).

Preparation of Wart Viral DNA for Electron Microscopy. To measure the contour length of wart viral DNA, the microdiffusion technique of Lang and Mitani (9) was used in a modification omitting formaldehyde (Bornkamm *et al.*,

Table 2. Molecular weights of fragments after cleavage with restriction endonucleases

DNA fragment	Molecular weight of DNA fragments ($\times 10^{-6}$)	
	WV-432/494	WV-474
RI A	4.10	4.10
RI B	0.63	0.63
Sum	4.73	4.73
Bam A	4.8	4.4
Bam B	—	0.45
Sum	4.8	4.85
Hind II A	2.25	2.25
Hind II B	1.80	1.80
Hind II C	0.78	0.78
Sum	4.83	4.83
Hind III A	3.75	3.75
Hind III B	0.90	0.85
Hind III C	0.23	0.23
Hind III D	—	0.07
Sum	4.88	4.90

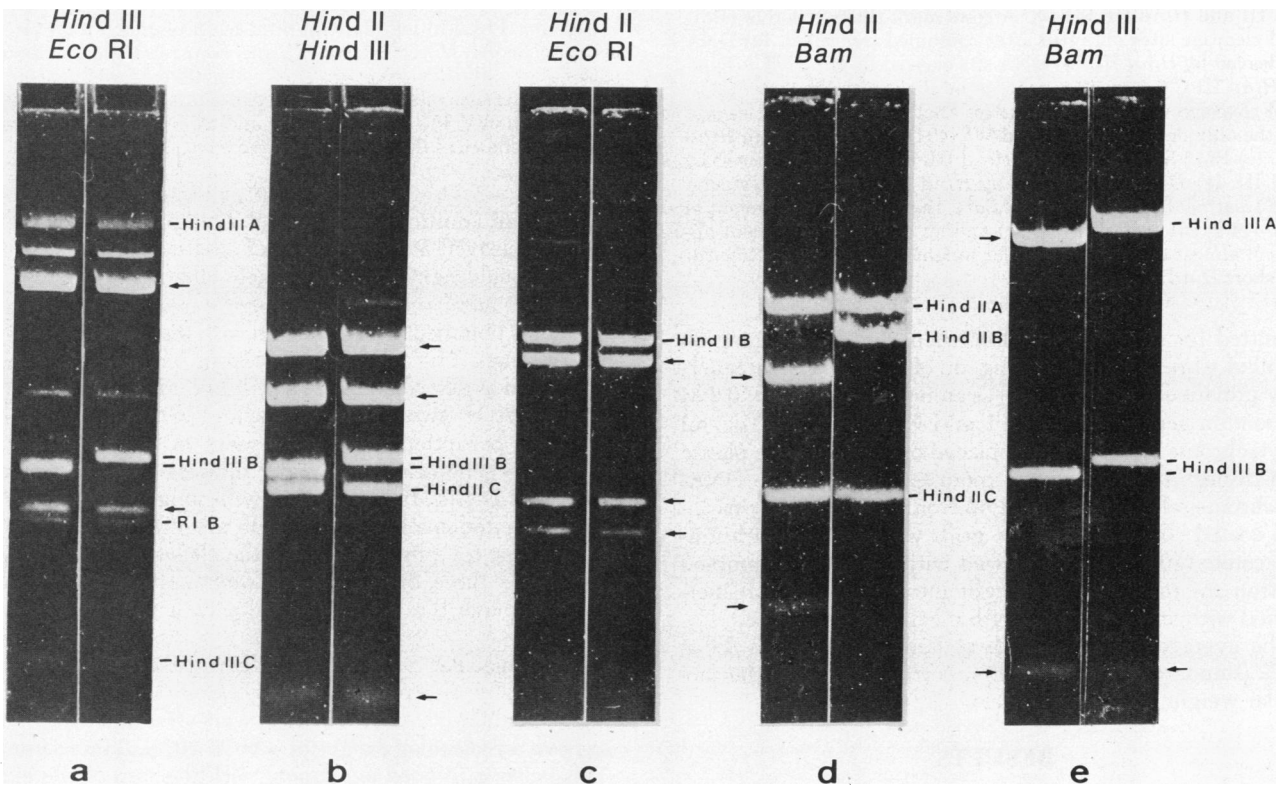

FIG. 1. Gel electrophoresis after restriction enzyme cleavage of DNA derived from WV-432 (right column) and WV-474 (left column). Arrows mark the position of additional cleavage sites obtained after combined treatment. (a) *Eco*RI + *Hind* III: fragment *Hind* III A is cut twice by *Eco*RI, resulting in three new fragments. One of them represents the complete *Eco*RI B fragment. Note that *Hind* III B of DNA 474 appears to be slightly shorter as compared to the same fragment in DNA 432, indicating that there exists a fourth *Hind* III cleavage site in the 474 genome. For the additional fragment, see Fig. 2. (b) *Hind* II + *Hind* III: this pattern shows that the *Hind* II cleavage sites are located within the *Hind* III A or *Hind* III C fragments. (c) *Hind* II + *Eco*RI: *Eco*RI cleaves the DNA within the *Hind* II A and *Hind* II C fragment. No difference between DNAs 432 and 474 can be seen. (d) *Hind* II + *Bam: Bam* cuts the *Hind* II B fragment of DNA 474 into two fragments. (e) *Hind* III + *Bam:* fragment *Hind* III A of DNA 474 is cut by *Bam*. The additional short fragment has almost the same molecular weight as *Hind* III C (see also Fig. 2). The results obtained with DNA 494 do not differ from those of DNA 432.

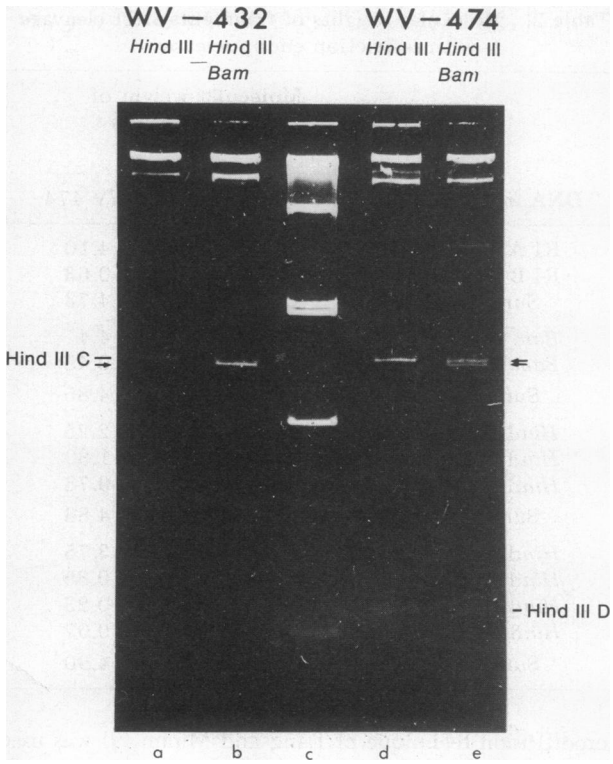

FIG. 2. Gel electrophoresis (4% polyacrylamide) after endonuclease cleavage of DNA derived from WV-432 and WV-474 with *Hind* III and *Hind* III + *Bam*. Arrows mark the position of additional cleavage sites obtained after combined treatment. (a) DNA 432 cleaved by *Hind* III. (b) DNA 432 cleaved by *Hind* III + *Bam*. The *Hind* III C fragment appears to be slightly shorter after additional cleavage with *Bam*, indicating that the first *Bam* cleavage site (the only one in WV-432 and WV-494) is located within *Hind* III C. (c) PM2 DNA cleaved by *Hind* III. (d) DNA 474 cleaved by *Hind* III. (e) DNA 474 cleaved by *Hind* III + *Bam*. After combined treatment with *Hind* III + *Bam*, the *Hind* III A fragment of DNA 474 is cut (see also Fig. 1e) and an additional fragment appears of almost the same molecular weight as *Hind* III C. Note the very short *Hind* III D fragment.

submitted for publication). Droplets of 50 μl containing 50 ng/ml of wart viral DNA, 50 ng/ml of lambda DNA (generously provided by W. Sugden) as an internal standard, 0.2 M ammonium acetate (pH 7.0), 1 mM EDTA, and 2.7 μg/ml of cytochrome *c* (Serva) were placed on hydrophobic plastic petri dishes. After 50 min at room temperature the DNA-cytochrome *c* film was picked up from the surface by parlodion coated copper grids. The grids were stained with uranyl acetate (10), rotary shadowed with Pt/P, and examined electron microscopically. Length measurements were performed with an automatic length measurement device.

The average contour length of lambda DNA was set at 100% (standard deviation 1.9%), corresponding to the molecular weight of 30.8×10^6 (11).

RESULTS

Cleavage of human papilloma virus DNA by five different restriction endonucleases resulted in the following pattern (Table 1):

(*i*) *Eco*RI cleaved at two sites in all three DNA preparations; (*ii*) *Hind* II treatment resulted in three fragments; (*iii*) *Sal* endonuclease appeared to have no cleavage site in all papilloma DNA preparations tested; (*iv*) *Hind* III treatment yielded three fragments in the DNA of WV-432 and WV-494, and an additional one in WV-474; and (*v*) *Bam* endonuclease cleaved WV-432 and WV-494 only once, whereas an additional fragment was obtained with WV-474 DNA.

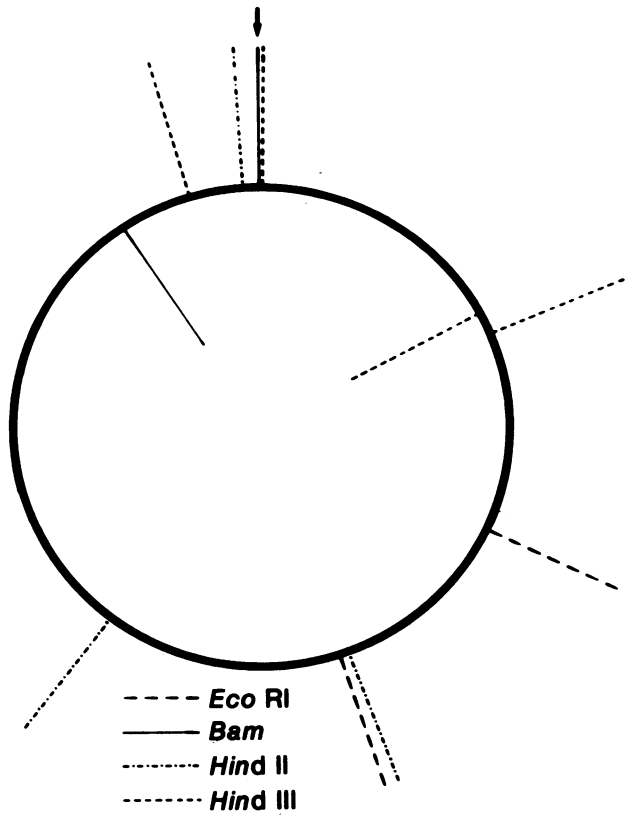

FIG. 3. Physical map of wart viral DNA obtained after cleavage with *Eco*RI, *Hind* II, *Hind* III, and *Bam* restriction endonucleases. The only *Bam* cleavage site in the 432 and 494 genome is used as reference site (arrow). The two additional cleavage sites obtained in WV-494 after *Hind* III and *Bam* treatment, respectively, are shown in the inner part of the circle.

Tables 1 and 2 show the number and molecular weights of fragments obtained after digestion with the different restriction enzymes. The calculation of the molecular weights was performed as described in *Materials and Methods*.

Cleavage by two different enzymes within the same experiment permitted the arrangement of single fragments within the genome. Figs. 1 and 2 show cleavage patterns of wart viral DNAs after treatment with some combinations of restriction endonucleases. The data are summarized in Fig. 3, revealing the physical map of the cleavage sites observed within the three differing papilloma virus DNA preparations. It shows the two additional sites within the DNA of WV-474.

The molecular weight of noncleaved viral DNA was determined by electron microscopy. Compared to bacteriophage lambda DNA (30.8×10^6), wart viral DNA was found to have a molecular weight of $4.86 \times 10^6 \pm 3.03\%$ (Fig. 4). This value is in good agreement with the sum of the cleavage fragments listed in Table 2.

DISCUSSION

With two different techniques (electron microscopic contour length measurement and agarose gel electrophoresis) the molecular weight of human papilloma virus DNA was calculated to amount to about 4.9×10^6. This value is some-

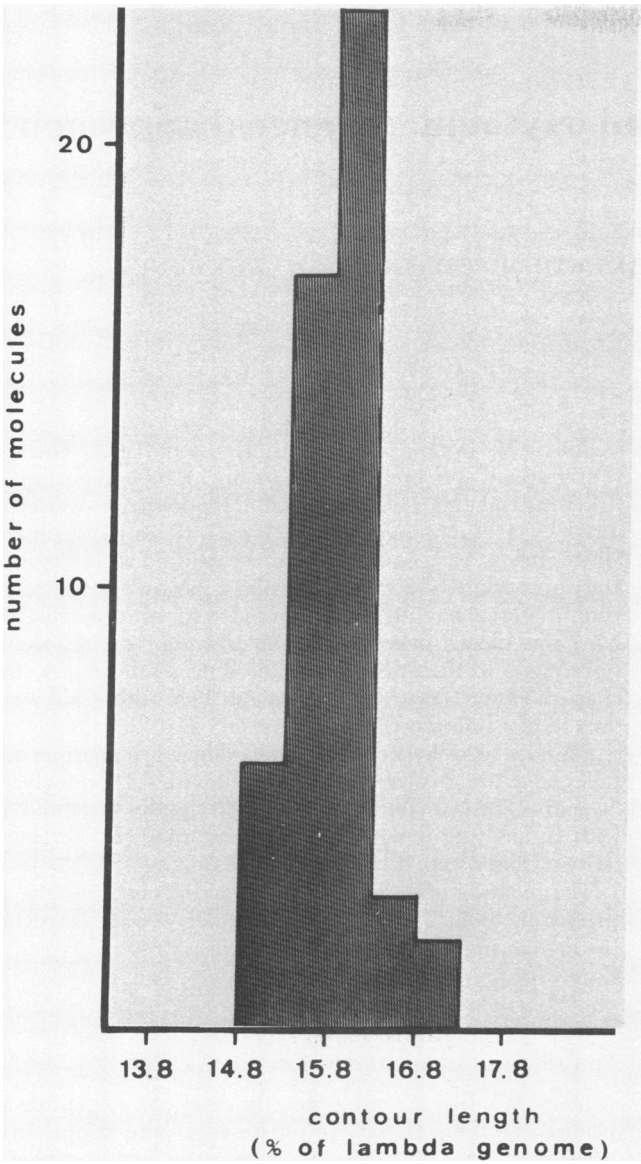

FIG. 4. Length distribution of wart viral DNA. Contour length is plotted as percentage of lambda DNA.

what lower than the one reported by Crawford (12), who determined a molecular weight of 5.3×10^6 by velocity sedimentation of papilloma DNA.

Analysis of papilloma virus DNA preparation after digestion with four different restriction endonucleases resulted in the physical map of the cleavage sites. One of the isolates tested contained one additional cleavage site after *Hind* III or *Bam* cleavage. This demonstrates that even isolates from the same kind of lesion (plantar wart) may reveal genetic heterogeneity. This heterogeneity is underlined by further data obtained with a fourth isolate (unpublished data). Based on agarose gel electrophoresis, the molecular weight of this DNA was calculated to amount to 4.6×10^6 only. Unfortunately, we did not obtain sufficient quantities of DNA to analyze its cleavage pattern.

These results thus provide evidence for genetic differences among human papilloma viruses. Additional differences in the epidemiological pattern of specific warts (5) and nucleic acid hybridization data (7) further support the existence of various types within a human papilloma virus group. Physical mapping of the DNA of specific isolates combined with serological methods should permit a type-specific characterization.

Note Added in Proof. After submission of this manuscript, physical mapping of human papilloma virus DNA was reported by Favre *et al.* (1975) *Proc. Nat. Acad. Sci. USA* **72**, 4810–4814. The data essentially conform with those reported here for WV-432 and WV-494.

We thank Dr. G. Schmid and Dr. W. Meinhof for providing the biopsy materials and Dr. B. Fleckenstein and Dr. G. W. Bornkamm for helpful suggestions. The skillful and devoted technical assistance of Mrs. U. Klenner is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 118: Methodenforschung zur Früherkennung des Krebses).

1. Goldschmidt, H. & Kligman, A. M. (1958) *J. Invest. Dermatol.* **31**, 175–182.
2. Serra, A. (1924) *Giorn. Ital. Mal. Venereol.* **65**, 1808–1814.
3. Ulman, E. V. (1923) *Acta Oto-Laryngol.* **5**, 317–334.
4. Rowson, K. E. K. & Mahy, B. W. J. (1967) *Bacteriol. Rev.* **31**, 110–131.
5. zur Hausen, H., Gissmann, L., Steiner, W., Dippold, W., & Dreger, I. (1975) *VII. Int. Symp. Comp. Leuk. Res., Copenhagen Oct. 13–17, 1975*, in print.
6. Almeida, J. D., Oriel, J. D. & Stannard, L. M. (1969) *Microbios* **3**, 225–232.
7. zur Hausen, H., Meinhof, W., Schreiber, W. & Bornkamm, G. W. (1974) *Int. J. Cancer* **13**, 650–656.
8. Tai, H. R., Smith, C. A., Sharp, P. A. & Vinograd, J. (1972) *J. Virol.* **9**, 317–325.
9. Lang, D. & Mitani, M. (1970) *Biopolymers* **9**, 373–379.
10. Davis, R. W., Simon, M. & Davidson, N. (1971) in *Methods in Enzymology*, eds. Grossmann, L. & Moldave, K. (Academic Press Inc., New York), Vol. 21, pp. 413–428.
11. Davidson, M. & Szybalski, W. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 45–82.
12. Crawford, L. V. (1965) *J. Mol. Biol.* **13**, 362–372.